Involvement of EGFR in the response of squamous cell carcinoma of the head and neck cell lines to gefitinib

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Abstract. Epidermal growth factor receptor (*EGFR*) gene mutations are associated with the sensitivity of non-small cell lung carcinomas (NSCLCs) to gefitinib, but such findings have not been reported in squamous cell carcinomas of the head and heck (SCCHNs). Accordingly, we determined whether *EGFR* gene expression and mutations correlate with the *in vitro* efficacy of gefitinib in SCCHN cell lines. EGFR status was analyzed in 16 different SCCHN cell lines by polymerase chain reaction (PCR) and direct sequencing for activating mutations, by real-time quantitative RT-PCR, and by Western blot analysis for RNA and protein expression. Using direct sequencing of PCR products from exons 18-23 of 9 SCCHN cell lines, we found a heterozygous *EGFR* mutation ($EGFR_{mut}$) with a 2607G›A transition in exon 20 (G/A genotype). The 9 different cell lines that showed this mutation also showed higher sensitivity (lower IC_{50} values) to gefitinib than cell lines with wild-type *EGFR* ($EGFR_{wt}$: G/G genotype) (p=0.016). EGFR protein levels correlated robustly (r=0.76) and significantly (p=0.0007) with *EGFR* mRNA levels and with IC_{50} values for gefitinib (r=0.65, p=0.0067). *EGFR* mRNA correlated with IC_{50} values (r=0.67, p=0.0046). Our conclusion was that the heterozygous and synonymous transition of the *EGFR* gene and low EGFR expression levels of mRNA and protein in SCCHN may be reliable predictors of high sensitivity in SCCHN patients to gefitinib.

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Introduction

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein receptor which exhibits a tyrosine kinase activity that regulates cell growth. EGFR overexpression has been frequently observed in squamous cell carcinomas of the head and neck (SCCHNs) and is thought to be involved in carcinogenesis and metastasis. Moreover, EGFR overexpression correlates with tumor size, clinical stage and poor prognosis (1-4). EGFR signaling has been found to control not only cell growth, but also angiogenesis and DNA repair (5,6), and has recently been assessed as a molecular target in the treatment of SCCHN.

Gefitinib (ZD1839, Iressa®), a low-molecular-weight anilinoquinazoline, is an orally active agent that reversibly inhibits EGFR tyrosine kinase activity. It has an acceptable toxicity profile and promising clinical efficacy in SCCHN patients in Phase I and II trials (7-10). Gefitinib is expected to be efficacious in non-small cell lung carcinoma (NSCLC) patients with high levels of EGFR expression because EGFR is also overexpressed in a significant portion of NSCLCs (11,12). However, results from clinical trials on the treatment of NSCLC with gefitinib have been varied and not statistically significant (13-15). A potential molecular mechanism underlying this variable clinical response is suggested by recent reports showing that kinase domain mutations of the *EGFR* gene in NSCLC tissues predict clinical responses to gefitinib (16,17).

In the present study, we determined whether *EGFR* gene expression and mutations correlate with *in vitro* efficacy of gefitinib in SCCHN cells.

Materials and methods

Reagents. Gefitinib was a gift from Astra-Zeneca Pharmaceuticals (Macclesfield, UK). RPMI-1640 was purchased from Nissui (Tokyo, Japan). Enhanced chemiluminescence (ECL) assay kits were from Amersham Biosciences (Piscataway, NJ). Protein assay kits were from Bio-Rad (Richmond, CA). SepaGene DNA purification kits were from Sanko Junyaku (Tokyo, Japan). TetraColor One MTT [3-(4,5-Dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide] assay kits were from the Seikagaku Corporation (Tokyo, Japan). Isogen RNA extraction kits were from Nippon Gene (Tokyo, Japan). TaKaRa RNA PCR Kits (AMV) Version 3.0 were from Takara Bio (Tokyo, Japan). TaqMan PCR Reagent Kits were from Applied Biosystems (Foster City, CA). RNase was from Becton-Dickinson (Franklin Lakes, NJ). The antibodies used were as follows: anti-EGFR (sc-03) polyclonal antibody (pAb) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-EGFR (1H12) monoclonal antibody (mAb) (1:1000), anti-p44/42 mitogen-activated protein (MAP) kinase pAb (1:1000), anti-phospho-p44/42 MAP Kinase pAb (1:1000), anti-Stat3 (124H6) mAb (1:1000) and anti-phospho-Stat3 (58E12) mAb (1:1000) (all from Cell Signaling Technology, Danvers, MA); anti-ß-actin (AC-15) mAb (1:10000) (Sigma Aldrich, St. Louis, MO); sheep anti-mouse IgG antibody (1:1000) and donkey anti-rabbit IgG antibody (1:1000) (GE Healthcare Bio-Sciences, Piscataway, NJ).

Cell lines and culture conditions. Sixteen different cell lines derived from human head and neck squamous cell carcinomas (SCCs) were used in the study. The origins of these cell lines were: oropharynx (YCU-M862, YCU-M911, KCC-M871), hypopharynx (YCU-H891), larynx (KCC-L871, YCU-L891), tongue (KCC-T871, KCC-TCM902, KCC-TCM903, YCU-T891, KCC-TCM901, YCU-T892 and KCC-T873), oral floor (KCC-OR891) maxillary sinus (KCC-MS871, YCU-MS861) (18,19). KB (human oral floor carcinoma cells) and A431 (human epidermoid cancer cells of the cervix) were purchased from the Riken Cell Bank (Tsukuba, Japan). All cell lines were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum and incubated at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

Western blot analysis. Whole cell homogenates were prepared for Western blot analysis with RIPA buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). Nineteen samples containing 10 μg protein were mixed with an equal volume of SDS sample buffer consisting of 4% SDS, 125 mM Tris-HCl (pH 6.8) and 10% glycerol, and then separated by 7.5 or 10% SDS-poly-acrylamide gel electrophoresis with prior heating at 95˚C for 5 min. The separated proteins were transferred onto PVDF membranes (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine and 20% methanol. The blotted membranes were then blocked with 5% BSA in Tris-buffered saline (TBS) and incubated with the appropriate primary antibodies. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were used as secondary antibodies. The presence of peroxidase was revealed by an ECL assay kit. Immuno-reacted bands were visualized by exposure to X-ray film (Kodak X-Omat AR; Eastman Kodak Co., Rochester, NY) and quantified by densitometric analysis using NIH Image Software. Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay kit. BSA was used as the standard.

Detection of EGFR mutations. Genomic DNA was extracted using a SepaGene kit. Exons 18-23 of the *EGFR* gene were amplified by polymerase chain reaction (PCR) using the specific primer sets listed in Table I (16). The PCR products were further analyzed by direct nucleotide sequencing using ABI PRISM 3100 DNA Analyzer (Applied Biosystems) in order to detect mutations.

Table I. Primer sets for *EGFR* mutation analysis.

Target exon	Sequence (5',3')
Exon 18	Forward: TCCAAATGAGCTGGCAAGTG
	Reverse: TCCCAAACACTCAGTGAAACAAA
Exon 19	Forward: GTGCATCGCTGGTAACATCC
	Reverse: TGTGGAGATGAGCAGGGTCT
Exon 20	Forward: ATCGCATTCATGCGTCTTCA
	Reverse: ATCCCCATGGCAAACTCTTG
Exon 21	Forward: GCTCAGAGCCTGGCATGAA
	Reverse: CATCCTCCCCTGCATGTGT
Exon 22	Forward: TGGCTCGTCTGTGTGTGTCA
	Reverse: CGAAAGAAAATACTTGCATGTCAGA
Exon 23	Forward: TGAAGCAAATTGCCCAAGAC
	Reverse: TGACATTTCTCCAGGGATGC

Table II. Primer sets for RT-PCR.

Target gene	Sequence (5',3')
EGFR	Forward: TTCGATGATCAACTCACGGAAC
	Reverse: GCCACCCATATGTACCATCGAT
EGFR (TaqMan)	Forward: FAM-TGCTGGATGATAGACGCAG ATAGTCGCC-TAMRA
	Reverse: FAM-ACCACCACGGCCGAGCGG-TAMRA
ß-actin	Forward: TGAGCGCGGCTACAGCTT
	Reverse: TCCTTAATGTCACGCACGATTT

Growth inhibition assay. Cells were seeded in 96-well plates at 1x10^4 cells/well in 100 μl of medium. Twenty-four h after seeding, cells were treated with various concentrations of gefitinib for 24 h. The inhibition of cell growth was assessed using the MTT colorimetric assay. Relative growth inhibition rates for the vehicle control were calculated and IC_{50} values were determined as the drug concentrations associated with 50% survival of cells.

Real-time quantitative RT-PCR. Total-RNA was extracted using Isogen RNA extraction kits according to the manu-

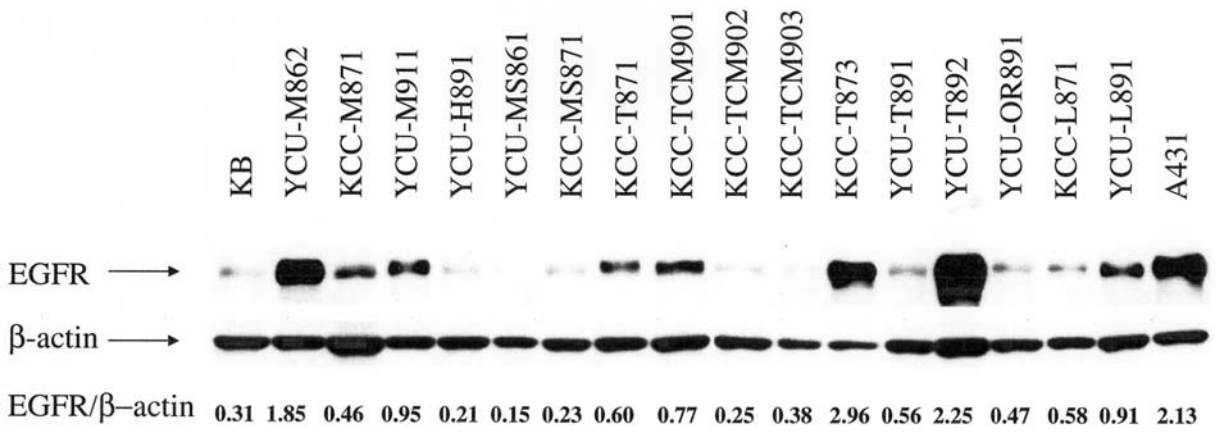

Figure 1. Expression of EGFR protein analyzed by Western blot analysis in 18 different cell lines. Expression levels of EGFR protein were determined by densitometry and relative expression levels by comparison with the expression levels of ß-actin.

facturer's protocol. Total-RNA (1 μg) was converted into cDNA using Takara RNA PCR Kits (AMV) Version 3.0. Real-time quantitative RT-PCR was carried out according to a fluorescence detection method using the TaqMan PCR Reagent kit or SYBG-based real-time PCR kit with specific primers, as shown in Table II. PCR was conducted using the following cycle parameters: 50˚C for 2 min and 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min.

Data analysis of quantitative RT-PCR. The threshold cycle (C_T) value of a target gene was divided by the endogenous reference (ß-actin) C_T value to correct for the input amount of cDNA. All corrected quantities were expressed as an n-fold difference relative to the gene expression levels of KB cells as the reference.

Results

Expression of EGFR protein and mRNA in SCCHN cell lines. We analyzed the expression of EGFR protein in 16 different SCCHN cell lines by Western blots (Fig. 1, Table III). Expression levels of EGFR protein in each cell line were determined by densitometry and by relative expression levels in comparison with the expression level of ß-actin. EGFR protein was detectable in all cell lines tested; levels were higher in KCC-T873 and YCU-T892 than in the A431 (positive control) cells.

Using real-time quantitative RT-PCR, we analyzed steady state levels of *EGFR* mRNA in 16 different SCCHN cell lines (Table III). *EGFR* mRNA was detected in all the cell lines tested. The steady state level of *EGFR* mRNA was highest in KCC-T873 cells and lowest in KCC-M871 cells, with the difference being >100-fold.

Comparison of IC_{50} values of gefitinib with EGFR expression and mutation. The drug concentrations, or IC_{50} values, used in each cell line and resulting in a 50% inhibition of cell growth are shown in Table III. KCC-MS871, KCC-TCM901 and KCC-T871 were the most sensitive to gefitinib, with IC_{50} values of 41.6, 43.9 and 44.4 μM, respectively.

Table III. Summary of EGFR expression and IC_{50} value for gefitinib.

Cell line	EGFR protein	*EGFR* mRNA	IC_{50} value (μM)
YCU-OR891	0.47	7.38	53.6
KCC-T871	0.60	0.89	44.4
KCC-T873	2.96	36.62	85.0
KCC-TCM901	0.77	0.15	43.9
KCC-TCM902	0.25	0.17	50.2
KCC-TCM903	0.38	1.21	51.4
YCU-T891	0.56	3.23	56.0
YCU-T892	2.25	7.70	64.8
YCU-MS861	0.15	0.17	66.3
KCC-MS871	0.23	1.38	41.6
YCU-M862	1.85	0.70	64.8
KCC-M871	0.46	0.09	52.1
YCU-M911	0.95	0.43	61.4
YCU-H891	0.21	1.04	69.0
KCC-L871	0.58	1.34	58.0
YCU-L891	0.91	4.23	52.2

Linear regression showed that, across cell lines, EGFR protein levels correlated robustly with *EGFR* mRNA levels (r=0.76, p=0.0007; Fig. 2A) and with IC_{50} values of gefitinib (r=0.65, p=0.0067; Fig. 2B). Steady state levels of *EGFR* mRNA also correlated robustly with IC_{50} values of gefitinib (r=0.67, p=0.0046; Fig. 2C). Although we found duplex bands of EGFR protein in some cases (see Fig. 5), EGFR protein levels did not have a significant correlation with any other parameters, including IC_{50} values.

Through the direct sequencing of PCR products from exons 18-23 of 9 SCCHN cell lines, we found a heterozygous *EGFR* mutation with a 2607G›A transition (*EGFR*mut) in exon 20.

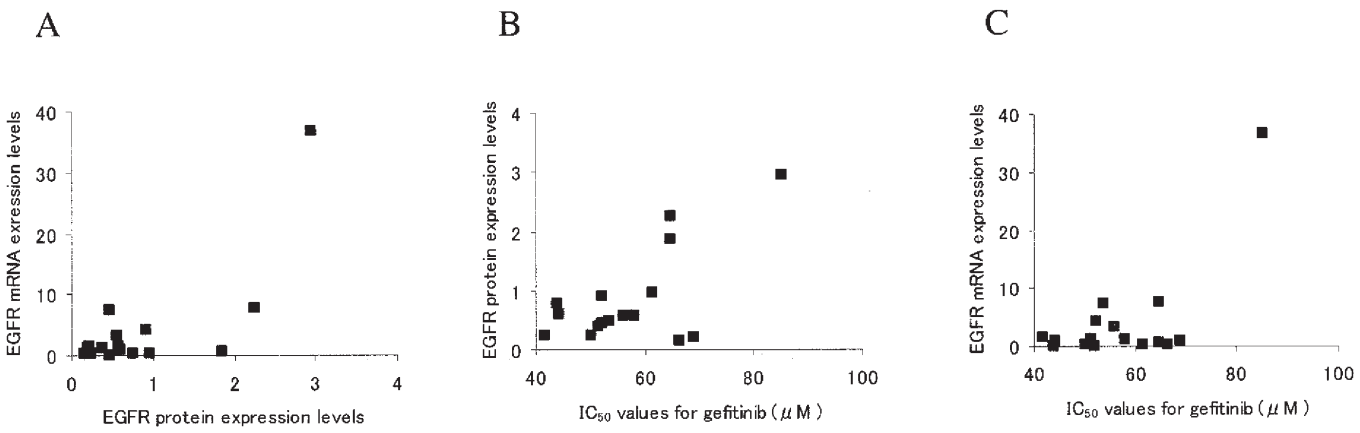

Figure 2. Response to gefitinib correlated to EGFR protein levels and *EGFR* mRNA levels. EGFR protein levels were determined by densitometric analysis of Western blot data as shown in Fig. 1. *EGFR* mRNA levels were quantified by real-time RT-PCR. (A) *EGFR* mRNA level vs. EGFR protein level (r=0.76, p=0.0007); (B) EGFR protein level vs. gefitinib IC_{50} (r=0.65, p=0.0067); (C) *EGFR* mRNA level vs. gefitinib IC_{50} (r=0.67, p=0.0046).

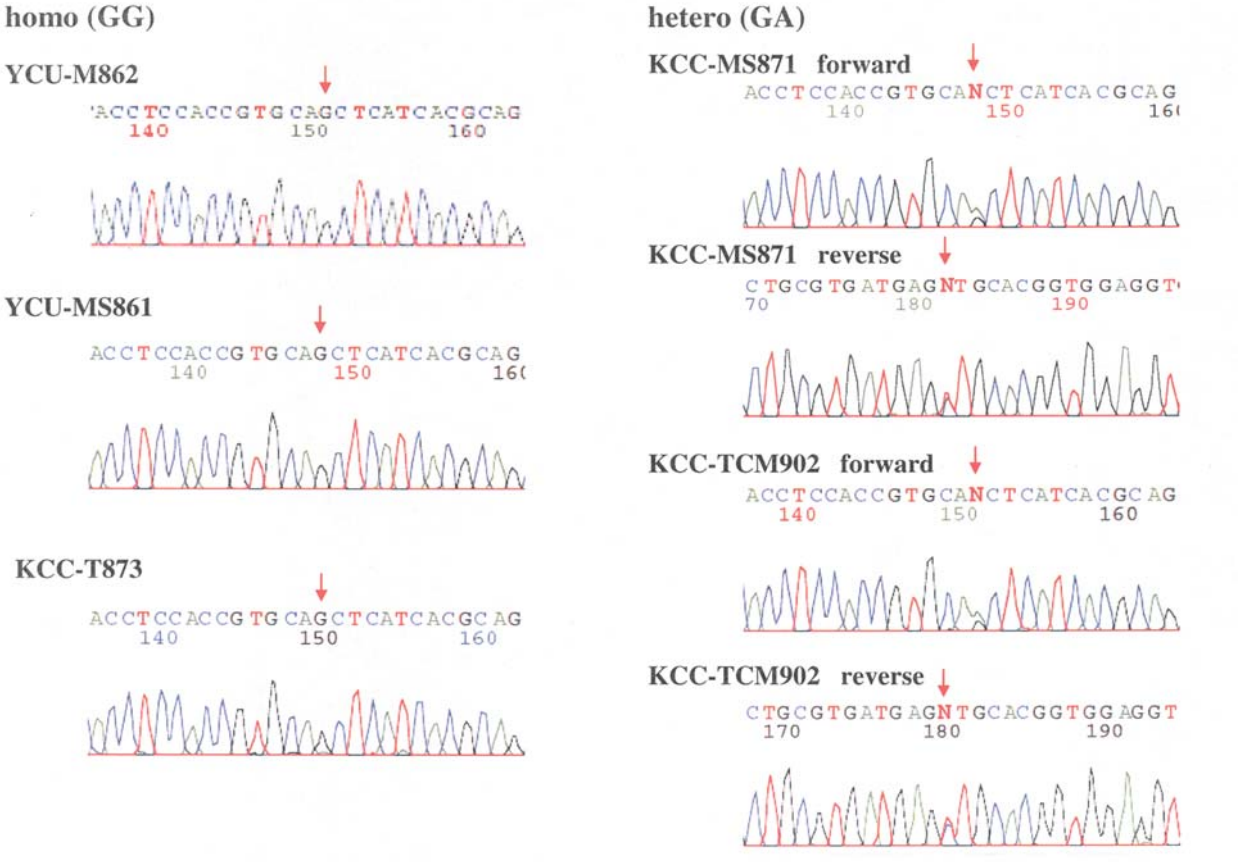

Figure 3. G/A genotype in *EGFR* gene. Genomic DNA was extracted and amplified with specific primers as shown in Table I. By direct sequencing of PCR products, single nucleotide polymorphisms (SNPs) (2607G›A transition, G/A genotype) in exon 20 were found in 9 cell lines. The G/G genotype as *EGFR*wt was found in 7 cell lines. Representative results are shown: G/G genotype, YCU-M862, YCU-MS861 and KCC-T873; G/A genotype, KCC-MS871 and KCC-TCM902.

This transition was synonymous (Q787Q) and identified with a known single nucleotide polymorphism (SNP) (rs1050171 in the NCBI SNP database) (Fig. 3, Table IV). We then compared the frequencies of the *EGFR* gene genotype with the IC_{50} values of gefitinib (Fig. 4). The heterozygous *EGFR*mut (2607G›A; G/A genotype) group, which was found in 9 different cell lines, showed a higher sensitivity (lower IC_{50} value) to gefitinib than did cell lines with wild-type *EGFR* (*EGFR*wt; G/G genotype) (p=0.016; Fig. 4C). However, differences in EGFR protein or mRNA expression levels were not significant between the G/G and G/A genotypes (Fig. 4A and B).

Table IV. Mutation in tyrosine kinase domain of *EGFR*.

Cell line	Exon 20 (2607)
YCU-OR891	G›A
KCC-T871	G›A
KCC-T873	WT
KCC-TCM901	G›A
KCC-TCM902	G›A
KCC-TCM903	G›A
YCU-T891	G›A
YCU-T892	WT
YCU-MS861	WT
KCC-MS871	G›A
YCU-M862	WT
KCC-M871	WT
YCU-M911	WT
YCU-H891	G›A
KCC-L871	WT
YCU-L891	G›A

WT, wild-type.

Effects of gefitinib on signaling downstream of EGFR. We examined the effects of gefitinib on the phosphorylation of EGFR, STAT3 and p42/p44 MAP kinase in 5 representative cell lines carrying *EGFR*wt (YCU-M862, YCU-MS861 and KCC-T873 cells) and heterozygous *EGFR*mut (GA genotype) (KCC-MS871 and KCC-TCM901 cells) (Fig. 5). Regardless of genotype, EGFR was phosphorylated by EGF stimulation within 24 h. Its phosphorylation was significantly suppressed by gefitinib treatment in all 5 cell lines. STAT3 phosphorylation by EGF stimulation at 50 μg/ml was only seen in the GG genotype cell line, YCU-M862, and this activation was inhibited by gefitinib. In the other 4 cell lines, STAT3 was constitutively active. This activation was not altered by either EGF and/or gefitinib treatment. p44/42 MAP kinase was phosphorylated by the stimulation of EGF. This phosphorylation was decreased by gefitinib treatment in all 5 cell lines. However, in the G/A genotype cell line KCC-TCM901, the inhibitory effect of gefitinib on MAP kinase phosphorylation was weak. As shown in Table III, the IC_{50} values of gefitinib in YCU-M862 cells (remarkable down-regulation of STAT3 phosphorylation by gefitinib) and KCC-TCM901 cells (insensitive to the MAP kinase-phosphorylation-inhibitory activity of gefitinib) were 66.3 and 43.9 μM, respectively. These data suggest that the inhibition of phosphorylated levels of p44/42 MAP kinase and STAT3 after gefitinib treatment are not predictors of a response to gefitinib.

Discussion

In earlier studies, the efficacy of gefitinib was thought to depend on EGFR expression levels in NSCLCs as compared to adjacent normal cells (12,20). However, this concept has recently been negated in other clinical trials. The most recent study showed that EGFR protein levels did not differ between SCCs and adenocarcinomas in lung cancer and that there were no significant correlations between EGFR expression and gefitinib IC_{50} values (21). Moreover, the report showed that NSCLC cell lines with high copy numbers of the *EGFR* gene were more sensitive to gefitinib treatment, and suggested that high copy numbers of EGFR are a benefit in gefitinib treatment (21).

Unlike studies on cell lines from lung cancers, the present study showed an association between expression levels of

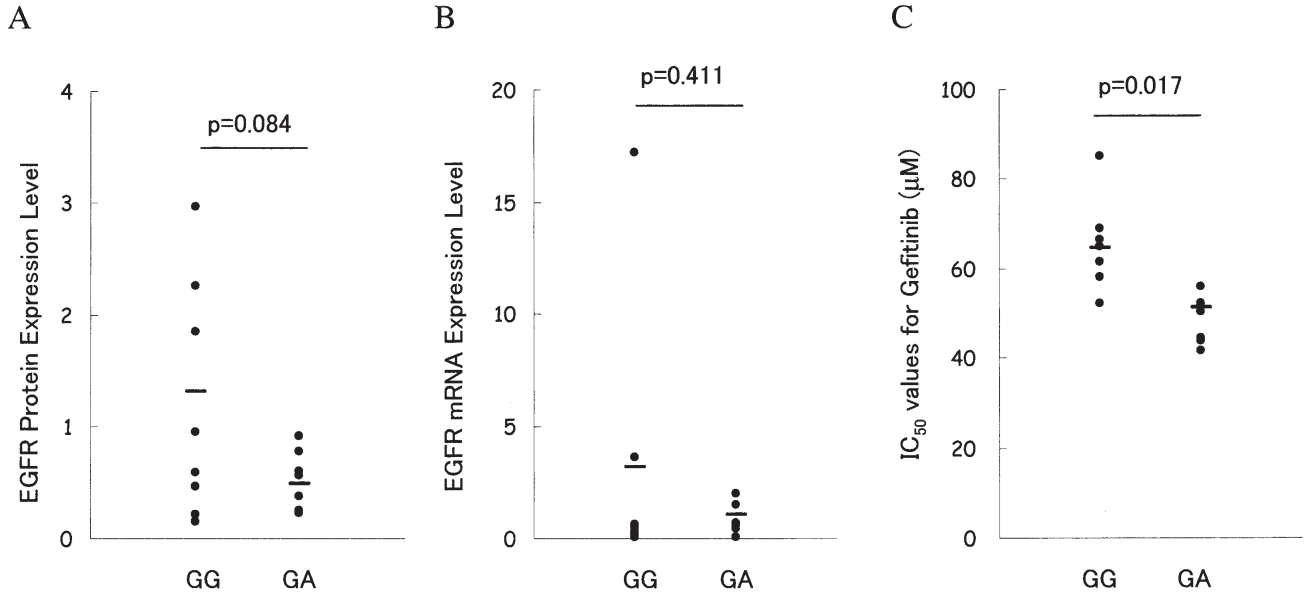

Figure 4. G/A genotype as an indicator of high response rate to gefitinib. G/G and G/A genotypes were compared in terms of (A) EGFR protein level, (B) *EGFR* mRNA level and (C) IC_{50} values of gefitinib. Cells with the G/A genotype showed higher drug sensitivity (lower IC_{50} values) to gefitinib than did those with the G/G genotype (*EGFR*wt). Statistical analysis, paired Student's t-test.

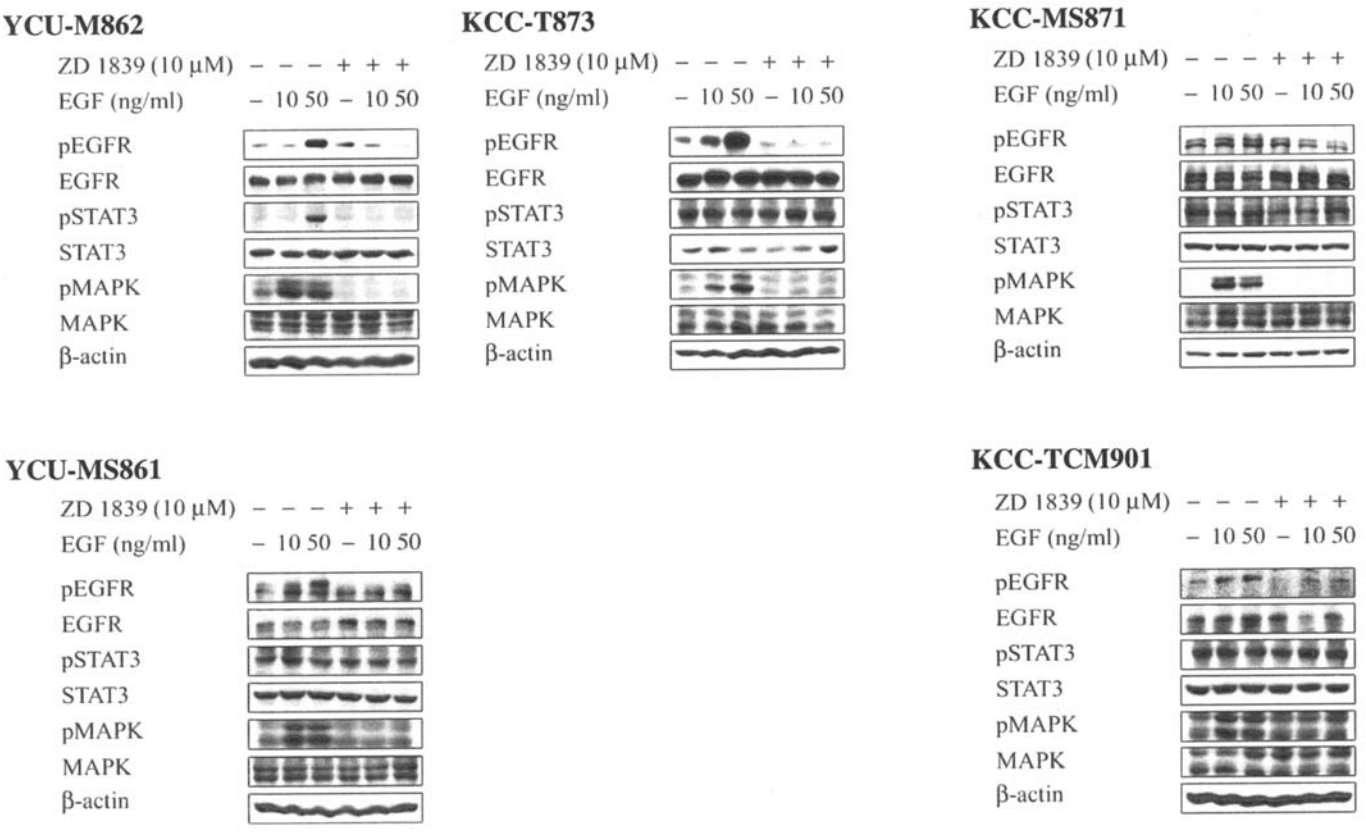

Figure 5. Effect of gefitinib on the downstream signaling of EGFR. The effects of gefitinib on the phosphorylation of EGFR, STAT3 and p42/p44 MAP kinase were examined in cell lines representative of *EGFR*wt (YCU-M862, YCU-MS861 and KCC-T873 cells) or heterozygous *EGFR*mut (GA genotype) (KCC-MS871 and KCC-TCM901 cells). EGFR was phosphorylated by EGF stimulation. Its phosphorylation was significantly suppressed by gefitinib treatment in all 5 cell lines. STAT3 phosphorylation by EGF stimulation at 50 μg/ml was only seen in the GG genotype cell line YCU-M862, and this activation was inhibited by gefitinib. The p44/42 MAP kinase was phosphorylated by stimulation with EGF. This phosphorylation was decreased by gefitinib treatment in all 5 cell lines. However, in the G/A genotype cell line KCC-TCM901, the inhibitory effect of gefitinib on MAP kinase phosphorylation was weak.

EGFR protein and gefitinib IC_{50} in cell lines from SCCHNs. However, we also showed that even SCCHN cell lines that are sensitive to gefitinib require higher concentrations (higher IC_{50} values) than those reported for NSCLC (22). This suggests that, in order to show clinical efficacy, the dose for patients with SCCHN might need to be higher than the approved dose (250 mg/day) for patients with NSCLC.

Several articles have revealed molecular mechanisms in which *EGFR* gene mutations are associated with altered sensitivity to gefitinib in NSCLCs (16,23,24). Mutations of the *EGFR* gene in the tyrosine kinase domain at exons 18-21 are one of the critical factors of gefitinib response in NSCLC (25). Of these mutations, 86% were found in 2 hotspot regions, exons 19 and 21, with the remaining 14% scattered throughout exons 18-21, whereas in SCCHNs it has been reported that *EGFR* mutations are predominant in exon 19 (22,26). EGFR mutations in the tyrosine kinase domain were common in Asian patients (7.3%) (26) and rare in Caucasian patients (1%) (22). We observed an SNP in exon 20 of 9/16 SCCHN cell lines tested (56.3%). This SNP, listed in the NCBI SNP database as rs1050171, is located in the EGFR tyrosine kinase domain. Due to a synonymous transition (Q787Q), it has been considered a silent mutation. The current frequencies of the genotype at this position are: European, A/A (37.5%), A/G (45.8%), G/G (16.7%); Asian, A/A (4.2%), A/G (20.8%), G/G (75.0%) (NCBI database, rs1050171). The efficacy of gefitinib against SCCHN in Caucasians is not significantly different than it is in Asians, although this genotype has a higher incidence of occurence in Europe. The presence of this SNP (G/A genotype) is therefore limited. However, it may be a beneficial parameter in Asian patients with SCCHN since, in the present study, the heterozygous *EGFR*mut (G/A genotype) group showed higher sensitivity (lower IC_{50} values) to gefitinib than the *EGFR*wt (G/G genotype) group did.

Dominant mutations observed in NSCLCs were the deletion of 2-15 nucleotides between codon E740 and 753 in exon 19, e.g., delE747_E749 and delE746_A750, and a point mutation, L858R, in exon 21 (25). These mutations increase the tyrosine kinase inhibitory activity of gefitinib due to conformational changes at the ATP-binding site. However, they were rare in SCCHNs in both our study and those of others (26). Our data show a good correlation between synonymous mutations (Q787Q) and response to gefitinib. It is thought that synonymous mutation Q787Q may not be 'silent', but may in fact result in abnormal splicing. For example, in Marfan syndrome a synonymous amino acid substitution (Q508Q) of *TGFBR2* (transforming growth factor-ß receptor 2) has been reported to occur because of abnormal splicing (27). The *tau* gene, whose encoded protein is associated with microtubule formation, has an S305S silent mutation which causes progressive supranuclear palsy pathology (28).

In some of the SCCHN cell lines tested in the present study, we found duplexes in the anti-EGFR antibody-reactive band. However, this alteration was not strongly correlated with either the frequency of the synonymous mutation or with the response to gefitinib. Alternatively, a synonymous mutation may affect the half-life of *EGFR* mRNA or EGFR turnover. Further studies are needed to clarify the effects of Q787Q mutations on the production of abnormal splicing variants.

In conclusion, we found that high expression levels of EGFR mRNA and protein are associated with low sensitivity to gefitinib. Furthermore, cell lines with heterozygous and synonymous transitions of the *EGFR* gene (Q787Q, rs1050171 (NCBI SNP database) have a significantly higher sensitivity to gefitinib than do *EGFR*wt cell lines. Thus, our data suggest new advantages in molecular-targeting therapy using gefitinib for selected patients with SCCHN.

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